RESEARCH ARTICLE

Latitudinal cline in weapon allometry and phenology of the European stag beetle

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Citation: Romiti F, Redolfi De Zan L, Rossi de Gasperis S, Tini M, Scaccini D, Anaclerio M, Carpaneto GM (2017) Latitudinal cline in weapon allometry and phenology of the European stag beetle. In: Campanaro A, Hardersen S, Sabbatini Peverieri G, Carpaneto GM (Eds) Monitoring of saproxylic beetles and other insects protected in the European Union. Nature Conservation 19: 57–80. https://doi.org/10.3897/natureconservation.19.12681

Abstract

Animal body size commonly exhibits a remarkable variation in response to environmental conditions. Latitude, when correlated with temperature, rainfall and seasonality, represents one of the main determinants of variation in body size, as well as in allometry. It has long been recognised that populations of larger body size are found in colder environments (Bergmann's Rule), a cornerstone of evolutionary ecology. However, the way in which latitude might influence investment in exaggerated weapons of animals has received little attention. The European stag beetle *Lucanus cervus* (Linnaeus, 1758) is the focus of this study. Males of this species exhibit exaggerated mandibles, mainly used as weapons during intra-sexual conflicts. Five populations ranging from northern Italy to the southern limit of the distribution of *L. cervus* were analysed. Combining morphological and phenological data, latitudinal variation in body size, weapon investment and activity period of the adults were evaluated. The analysis of the allometry of mandibles strongly supported the presence of two male morphs. Large males (major morph) invest significantly more in weapons compared to males of the minor morph. Consistent with Bergmann's Rule, these results confirmed that

the stag beetle body size increased at higher latitudes (N) and that this increase in size triggers an arms race which leads to further exaggeration of male weapons which is particularly evident in major males. In this morph, the mandible allometric coefficient line was steeper for the northern populations. The activity period also varied with latitude, beginning later at lower latitudes. Characterisation and comparison of adult phenologies provide valuable data to be used in the design of monitoring programmes for this threatened species and are important for modelling the species responses to climate change.

Introduction

Variation in animal body size has always attracted considerable interest from biologists. Populations and species can exhibit remarkable variation in body size, as well as in other traits, in response to different environmental conditions (Stillwell 2010). One of the major reasons for variation in environment and ecology is variation in latitude, with corresponding variation in temperature, rainfall and seasonality. The latitudinal clines in body size, described for endotherms, have interested biologists since it was recognised by Bergmann (1848). Bergmann's Rule states that, within a widespread taxonomic group, populations, species and genera of larger body size are found in colder environments, usually located at higher latitudes, while the opposite is observed in warmer environments, usually situated at lower latitudes. In many organisms, individuals living in colder environments grow more slowly but attain larger size as adults (Angilletta and Dunham 2003). Latitude also correlates with resources availability which influences the development time of ectotherms (Arnett and Gotelli 1999). First recognised and used with endothermic animals, Bergmann's Rule has been investigated widely in ectothermic organisms (e.g., crustaceans (Timofeev 2001); insects (Cushman et al. 1993); fish (Belk and Houston 2002); amphibians (Adams and Church 2008, Ashton 2002); reptiles (Ashton and Feldman 2003)). Much debate has arisen regarding the formulation and definition of Bergmann's Rule and this continues to the present day. Does it deal with inter- or intraspecific differences and is it valid for ectothermic organisms (Shelomi 2012)? As ectotherms rely on external sources for body heat, environmental temperature is particularly important and often correlated with the difference in body size (Atkinson 1994). Bergmann's Rule concerning body size clines in ectotherms has given rise to some doubts about its validity and has led to heated debates (e.g., Van Voorhies 1996, 1997, Mousseau 1997). Indeed, a general consensus has not been reached to support the Bergmann's Rule for ectotherms: some authors report the presence of the converse-Bergmann (e.g., Schutze and Clarke 2008), others have stated that Bergmann's Rule cannot be considered a valid eco-geographical law for insects (Shelomi 2012), or Bergmann and converse-Bergmann could not even be mutually exclusive, being driven by different environmental causes (Blanckenhorn and Demont 2004). Using common garden experiments, several studies on insects (Anderson 1973, Cavicchi et al. 1985, 1989, Partridge et al. 1994) proved that body

size increases in low-temperature conditions and that selection favours larger body size at low temperatures (McCabe and Partridge 1997, Reeve et al. 2000). Furthermore, several studies which compared geographical variation and biometric traits of insects, have recorded a latitudinal correlation with body size (Capy et al. 1993, Cushman et al. 1993, Arnett and Gotelli 1999, Huey et al. 2000, Stillwell et al. 2007). Recently, in studying the exaggerated rostrum of the New Zealand giraffe weevil *Lasiorhynchus barbicornis* (Fabricius, 1775), Painting et al. (2014) linked the latitudinal cline in body size with allometry (*sensu* Huxley and Teissier 1936). The European stag beetle, *Lucanus cervus* (Linnaeus, 1758), like many other species of Lucanidae, shows remarkable sexual dimorphism in mandible size and shape (Franciscolo 1997) and a great variation amongst males for this characteristic (Clark 1977, Harvey et al. 2011, Hardersen et al. 2011, Romiti et al. 2015, 2016). The mandibles of *L. cervus* males can be considered as secondary sexual characteristics (SSC) that are used as weapons in intra-sexual combats (Darwin 1871, Arrow 1951, Mathieu 1969, Otte and Staymen 1979, Lagarde et al. 2005). Knell et al. (2004) recognised two trajectories for the allometric relationship between mandible length and body size in different species of the genus *Lucanus*. Species with relatively short mandibles exhibit a steeper allometric coefficient (slope of the regression) as size increases; on the other hand, a limit for the development of such exaggerated structures is evident in species with relatively long mandibles. *L. cervus* males use their mandibles in male-to-male combat (Clark 1977, Lagarde et al. 2005, Fremlin 2009) and major males are equipped with enlarged and more complex mandibles (Romiti et al. 2015, 2016). The analysis of the geographical variation of allometric trajectories allows researchers to obtain more robust conclusions about the relative investment in these SSC. Size and shape of SSC are likely to be the result of sexual selection (West-Eberhard 1983, Andersson 1994, Jennions et al. 2001, Stuart-Fox and Ord 2004) and typically exhibit high levels of developmental plasticity and condition dependence (Cotton et al. 2004), probably as a result of changes in environmental and individual conditions (Cassidy et al. 2014) within and amongst populations. Consistent with Bergmann's Rule and considering *L. cervus* as a stag beetle species with relatively short mandibles (*sensu* Knell et al. 2004), it was expected that increasing the latitude would increase the allometric coefficient, as more resources would likely be allocated to the development of the weaponry of the male stag beetles. Romiti et al. (2016) showed that there was no significant variation in the shape of the SSC of the stag beetle male amongst populations at the same latitude in central Italy. To the best of the authors' knowledge, only Painting et al. (2014) have investigated the latitudinal allometric cline of SSC in a beetle species and its correlation with body size variation.

The ecology of ectotherms is particularly affected by latitude as temperature has a strong influence on their biology (Willmer et al. 2000, Sperry et al. 2010). *L. cervus* is a saproxylic beetle, its larvae feed on and grow in dead decaying wood, while the pupal stage and eclosion occur near to the larval development site (Harvey et al. 2011, Speight 1989, Alexander 2008, Nieto and Alexander 2010). Although insects living within tree trunks, roots or logs are usually less sensitive to the photoperiod, their life

cycle (at least during the wood-boring stage) is governed by temperature fluctuations (thermoperiod) (Riba 1976). The majority of insects show daily and annual cycles of activity (phenology) related to climatic change caused by the earth's movements (Saunders 2002). Understanding the phenology of the adult stag beetle is vital if effective monitoring protocols are to be developed, an urgent requirement especially for species of conservation concern. *L. cervus* is considered a flagship species for conservation of saproxylic fauna. This species is listed in the European Habitats Directive 92/43/EEC (Appendix II), in the IUCN red list of saproxylic beetles as Near Threatened at European level (Nieto and Alexander 2010) and as Least Concern in Italy (Carpaneto et al. 2015), due to its sensitivity to dead wood management and wood exploitation (Audisio et al. 2014). For this reason, several efforts have been made at European level to understand the phenology of the species (e.g.,Vrezec 2008, Chiari et al. 2014) and to correlate the adult peak activity with geographical variables (Campanaro et al. 2016). Thus, morphological and phenological data have been combined (i) to assess whether there was a significant latitudinal variation in body size and weapon allometry in *L. cervus* males and (ii) to acquire information on adult stag beetle phenology across its Italian distribution.

Materials and methods

Sampling design and study areas

A total of 767 males of *L. cervus* were sampled during the breeding season between May and August in the summer 2012–2016. Captures were performed with an entomological net (Ø = 50 cm, telescopic handle = 2 m), by hand and with emergence traps placed on possible oviposition sites detected by means of radio-telemetry. Sampling activities were undertaken in five localities of Italy (Figure 1). These sites cover a latitudinal range of 2.57° which spans from the northernmost to the southernmost limit of the Italian distribution of the species. A total of 111 males were sampled in Monterosso (hereafter MON: 45.944568N, 8.524431E) from 2nd June to 12th July 2012. During two consecutive years, 2014 and 2015, 83 males were sampled in Bosco Fontana (hereafter BOF: 45.198434N, 10.747084E) from 29th May to 17th July 2014 and from 25th May to 4th July 2015. During three consecutive years, 2014–2016, 141 males were sampled in the Croara forest (hereafter CRO: 44.922222N, 9.575000E), from June to August (Scaccini and Anaclerio 2016). A total of 75 captures were performed in Tocerano (hereafter TOC: 43.440278N, 12.187222E) from 7th July to 4th August 2014. In Felceto (hereafter FEL: 43.376404N, 12.275146E), a total of 357 stag beetles were sampled during two consecutive years, 2014 and 2015, from July to August. Starting from the northernmost site, MON is a hill (690 m a.s.l.) characterised by an old-growth chestnut (*Castanea sativa*) woodland on the banks of Lake Maggiore; BOF is a relict forest of the Po plain (26m a.s.l.), dominated by common oak (*Quercus robur*)
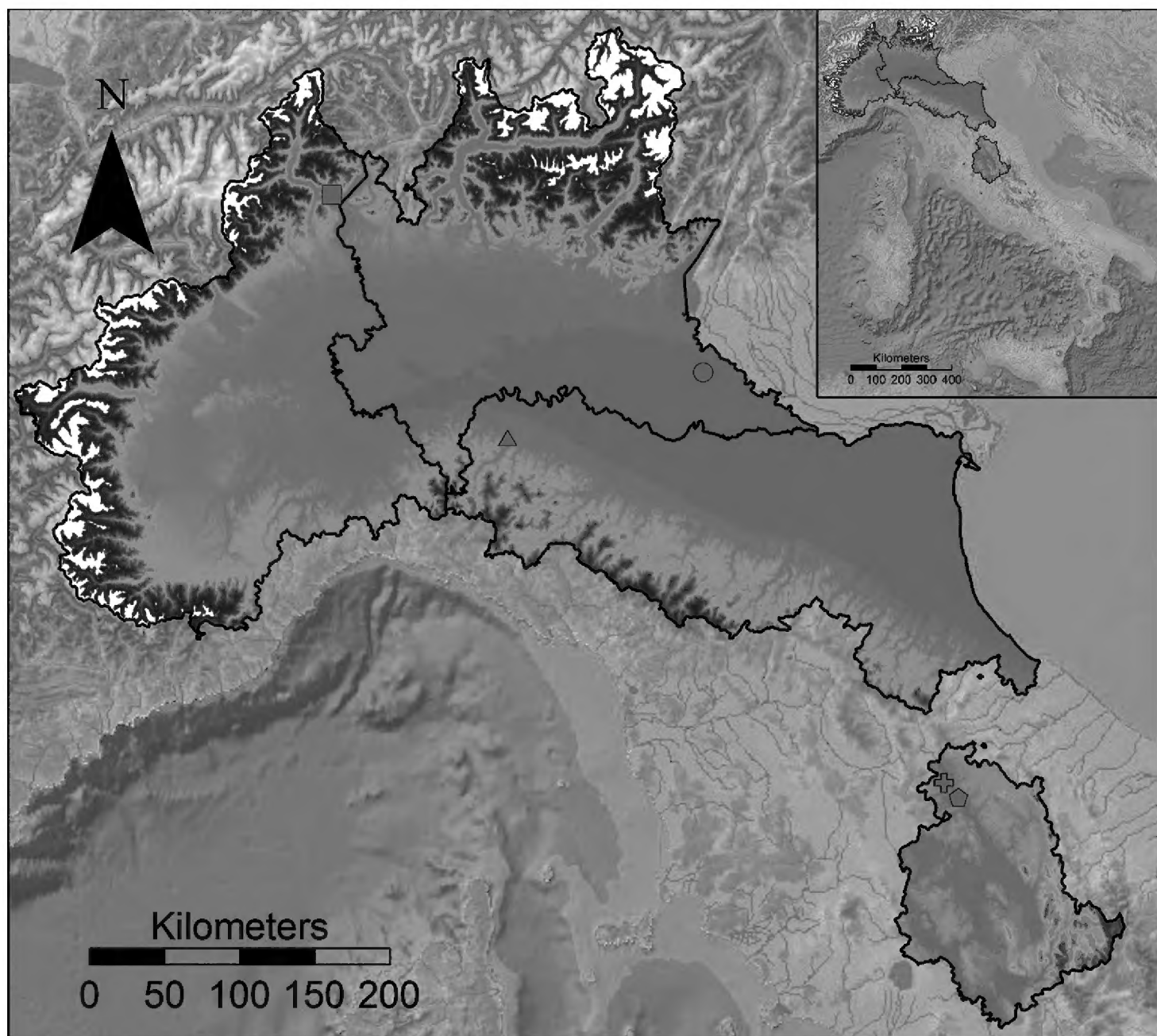
Figure 1. Overview of the study sites in five localities in Italy. Square: Monterosso (MON), Piemonte Region; Circle: Bosco Fontana (BOF), Lombardia Region; Triangle: Croara (CRO), Emilia-Romagna Region; Cross: Tocerano (TOC), Umbria Region; Pentagon: Felceto (FEL), Umbria Region.

and hornbeam (*Carpinus betulus*), with some introduced species such as the common red oak (*Quercus rubra*); CRO is a relict floodplain forest along the Trebbia river (153m a.s.l.), dominated by oaks (e.g., *Q. petrea, Q. cerris*) and chestnut (*C. sativa*) alongside sub-mediterranean species (e.g., *Fraxinus ornus, Sorbus torminalis, Q. pubescens*); TOC is a wooded hill (660m a.s.l.) characterised by mixed-oak forest and some cultivated fields and FEL is a wide clearing within a wood of Turkey oak (*Q.cerris*) along the Tiber river (332m a.s.l.). Each captured individual was marked, measured and weighed before being released. Linear body measurements were taken with an electronic caliper (precision 0.05 mm) and body mass (BM) was measured using a dynamometer (PE-SOLA AG, Switzerland) (precision 10mg). BM was not measured for beetles sampled in CRO. Mandible length and elytron length were measured as described in Chiari et al. (2014), Romiti et al. (2015) and Scaccini et al. (2016). Mandible length (ML) was measured as the linear distance from clypeus to the apex of the outer tooth of the fork

and this can be considered a good proxy for the actual mandible size (Romiti et al. 2016). The elytron length (EL) was preferred to the most commonly used body length to avoid errors caused by the mobility of segments (Juliano 1986). After removing outliers, a total of 763 male stag beetles (pooled dataset) were obtained, divided as follows: 109 for MON, 82 for BOF, 140 for CRO, 75 for TOC and 357 for FEL.

Climate data

Climate data were obtained for the study sites from WorldClim – Global Climate Data site (http://www.worldclim.org/). Mean (MEA), minimum (MIN) and maximum (MAX) monthly temperatures (C°) and monthly total precipitations (PRE) (mm) of the study sites were downloaded according to their coordinates using the geographic coordinate system WGS84 (not projected) in decimal degrees. The data provided by the web site (section "Current") are interpolations of observed data, representative of fifty years (1950-2000) and thus represent monthly averages of the selected variables. Data were downloaded with the highest spatial resolution available: 30 seconds, which correspond to cells of 0.86 km^2 at the equator (often referred as 1-Km spatial resolution) (Hijmans et al. 2005).

Data analysis

All the biometric variables were natural log transformed (Ln) and checked for normal distribution, as well as the climate variables and phenology, using Shapiro-Wilk normality test, prior to applying any parametric test.

Climatic characterisation

To analyse the variance in climate variables between sites, ANOVA and Tukey HSD tests were performed. The correlation between latitude and climate variables was investigated with the Pearson correlation test. Two discriminant (or classification) techniques were used to categorise sites into groups taking into account their climate variables as predictor characteristics. Both the principal component analysis (PCA) and the linear discriminant analysis (LDA) were applied to the sites' climate. PCA tries to retain most of the variability in the data, whereas LDA (MASS R package) looks for the combination of the climate variables that give maximum separation between the centres of the site data, minimising the variation within each site. The data were transformed, centred and scaled (caret R package) prior to the application of LDA (Kuhn and Johnson 2013). The results were then compared via scatterplots and inspection of the explained variance by the LDA and PCA axes was undertaken.

Morphometry and allometry

For the allometric analysis of male mandibles, the elytron length was used as an independent variable, mainly for three reasons: (i) its proven high correlation coefficient with mandible length (Romiti et al. 2015); (ii) the comparability with previous morphometric studies which used linear distances for *L. cervus* (Harvey and Gange 2006, Hardersen et al. 2011, Chiari et al. 2014, Romiti et al. 2015, Scaccini et al. 2016) and (iii) to avoid scale dependence issues in comparing traits with different units (e.g., mm vs g) which could affect the data despite the log transformation (Warton et al. 2006). The pooled dataset was used to analyse the allometric relationship between the mandible length (LnML) and the elytron length (LnEL) of *L. cervus* males. The scaling relationship between these traits was analysed using the linear and segmented regression models (segmented R package, Muggeo 2003). The Davies test (Davies 1987) was used to check for a change in slope in the fitted linear model. Other non-linear regressions (e.g., sigmoidal, gaussian) were not undertaken according to Romiti et al. (2015) which demonstrated the goodness of fit for a segmented model in describing the allometric relationship between mandible and body size. The same models were used for the analysis of intraspecific allometry. The models were compared on the basis of their "goodness of fit" using Akaike's information criterion (AIC) and ANOVA to determine which model gave the best description of the scaling relationship. Models which differed by <2 in the AIC score were considered to be indistinguishable from each other in their explanatory power (Burnham and Anderson 2002). To test how this scaling relationship varies across populations the "SMA function" (SMATR R package, Warton et al. 2012) was used, considering the population as the variable factor. SMA uses standardised major axis (SMA) and major axis (MA) estimations to evaluate the line of best fit and the confidence intervals for both slope (allometric coefficient) and elevation (proportionality coefficient) of each population and to test for significant differences in these regression parameters. The null hypotheses were, respectively: slopes are equal, no difference in elevation. The allometric coefficients (slope of the regression) of minor males, often close to 1, were tested for isometry (H0: slope not different from 1). To investigate whether stag beetle males exhibit differences in BM (excluding Croara population), ML and EL amongst populations, the biometric rough variables were used and tested with the Kruskal-Wallis test and Dunn's test for multiple comparisons (with Bonferroni correction). A PERMANOVA using Euclidean distance matrices (n. perm. = 999) was performed comparing biometric variables amongst different latitudes.

Phenology

To investigate the phenology of the species in each site, it was considered that the adult activity period lasted from the first day of capture to the last one. For the sampling sites with more than a year's field work (i.e., for BOF, FEL and CRO), the first and last cap-

ture date were taken into account, irrespective of the year. In this way, the phenology was dependent on site, rather than be year-dependent. Dates were then transformed into a number in Microsoft Excel 2010, maintaining a fictitious year to avoid any transformation error. Otherwise, inputting the same date for the different years, for example 25/05/2015 and 25/05/2014, different numbers could have resulted i.e. 42149 and 41784 respectively. To test if the phenology varies with latitude, the Kruskal-Wallis test and the Mann-Whitney post-hoc test for pairwise comparison were used.

Results

Despite the Ln transformation of the biometric variables, some maintained a non-normal distribution ($MON_{N = 109}$: LnML, $w = 0.972$, $p = 0.020$; $BOF_{N = 82}$: LnML, $w = 0.958$, $p = 0.009$; $TOC_{N = 75}$: LnBM, $w = 0.960$, $p = 0.019$; LnEL, $w = 0.964$, $p = 0.033$; $FEL_{N = 357}$: LnBM, $w = 0.991$, $p = 0.021$; LnML, $w = 0.966$, $p < 0.01$). Climate variables and phenological data were normally distributed (See Suppl. material 1: Table 1).

Climatic characterisation

Amongst the analysed climate variables, the monthly total precipitations showed a positive correlation with latitude (Pearson's test: $r = 0.32$, $p = 0.013$). The ANOVA results indicated no significant differences between sites as regard their monthly temperatures (MEA: DF = 4, $f = 0.29$, $p = 0.88$; MIN: DF = 4, $f = 0.32$, $p = 0.86$; MAX: DF = 4, $f = 0.28$, $p = 0.89$), but a significant variation in the variance of the monthly total precipitations (PRE: DF = 4, $f = 6.56$, $p < 0.01$). The Tukey HSD test indicated a clear difference in the total monthly precipitations ($p_{MONvsBOF} < 0.01$, $p_{MONvsCRO} < 0.01$, $p_{MONvsFEL} < 0.01$, $p_{MONvsTOC} < 0.01$), with the highest values recorded in MON (See Suppl. material 2: Figure 1). The first 3 PCs of PCA (Figure 2a) explained the total variance between sites (Table 1a), considering all the climate variables. The first 3 linear discriminants of LDA (Figure 2b) explained the total variance of the sites (Table 1b). Moreover, as a result of the great difference between sites regarding their PRE (See Suppl. material 2: Figure 1, thermo-pluviometric diagrams), the posterior probabilities (*pp*) of LDA indicated that most interpolated climate data of MON were assigned with high certainty to MON (*pp* mean %: 68%); whereas some uncertainties remained in the assignment of their own interpolated climate data to the other sites (*pp* mean %: BOF = 10%; CRO = 11%, FEL = 10%; TOC = 1%).

Morphometry and allometry

The result for the pooled dataset regarding the scaling relationship between mandible length (LnML) and elytron length (LnEL), indicated the presence of a switch point at
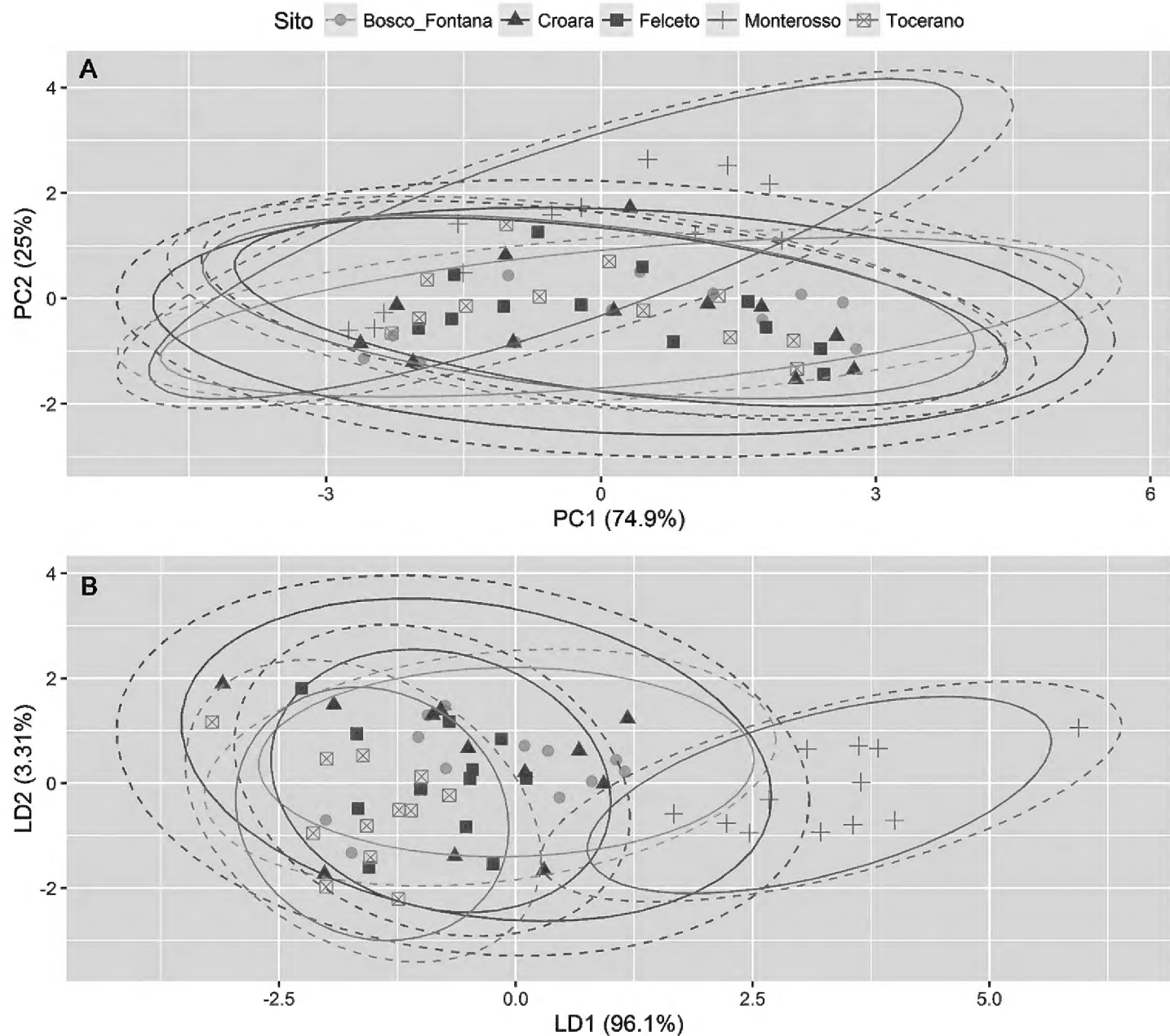
Figure 2. Scatterplots for the variation of climate variables amongst the sites: **A** first two principal components of the PCA **B** first two linear discriminants of the LDA. The percentage of variance explained by each axis was reported in brackets.

22.19 mm of elytron length (± 1.01 mm) (Table 2A). Indeed, the best fit model was the segmented regression which statistically differed from the linear model, as confirmed by the Davies test (k = 1000, p< 0.001) and which highlighted a switch point at 3.10 of LnEL by the ANOVA and AIC scores comparison (Table 2A). The scatterplot in Figure3 illustrates the scaling relationship between LnML and LnEL for minor and major males, with the steepest allometric coefficient line for those belonging to the major morph. With regard to the intraspecific allometry of the male mandible, the segmented regression resulted in the top ranked models for four of the five analysed populations. For the population of BOF, the linear and segmented model did not differ significantly, considering both the ANOVA result and the ΔAIC for the two models (Table 2B). In all the populations with segmented regression as the top ranked model, males of the minor morph exhibited a lower value for the allometric coefficient compared to major males. The results of the SMA function revealed a significant variation in the allometric coefficient between weapon and body size for major males (above

Table I. Summary of the results of the first 3 principal components (PC) and linear discriminants (LD) of principal component analysis and linear discriminant analysis, respectively.

| A	PC1	PC2	PC3
Standard deviation	1.7303	0.9998	0.0797
Proportion of Variance	0.7485	0.2499	0.0015
Cumulative Proportion	0.7485	0.9984	1
B	LD1	LD2	LD3
Proportion of trace	0.9197	0.0712	0.0090
Cumulative Proportion	0.9197	0.9909	0.9999
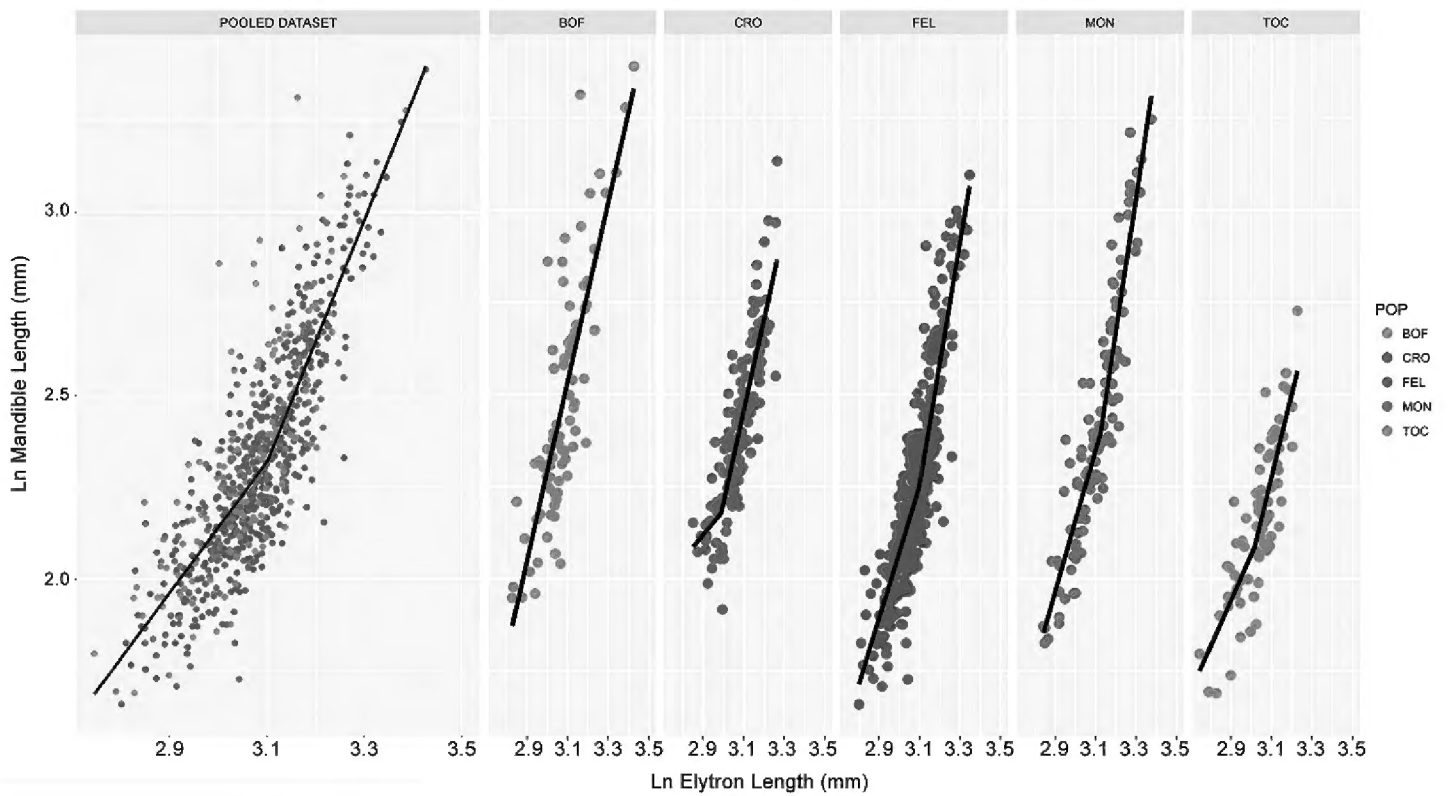
Figure 3. Scatterplots of the allometric relationship between mandible (LnML) and elytron (LnEL) length, with the top ranked model superimposed on the basis of its AIC score. The pooled dataset illustrates the trend of the scaling relationship for all the analysed populations. The allometric trajectory of each population was reported in subsequent scatterplots.

the estimated switch point) (DF = 4, LR = 66.00, $p < 0.01$). However, minor morph males did not differ in allometric coefficient between populations (SMA: DF= 4, LR = 5.23, $p = 0.26$), but exhibited a significant difference from isometry (SMA: DF = 351, r = 0.85, $p < 0.01$), also indicating the presence of a positive allometry for this morph. The allometric relationship between LnML and LnEL for minor and major males is illustrated in the scatterplots (See Suppl. material 3: Figure 2). The results for differences in elevation between populations indicate an overall significant difference (DF = 4, Wald = 207.2, $p < 0.01$). A non-significant difference in elevation was detected for populations at the same latitude (i.e., TOC and FEL), as a result of the multiple comparison amongst groups (TestStat = 1.19, $p = 0.28$) (See Suppl. material 4: Figure 3).

Table 2. Summary of the models results, obtained by fitting segmented and linear regressions to the scaling relationship between mandible length and elytron length for the pooled dataset (**A**) and for each population separately (**B**).

|	Dataset	Model	AIC	ΔAIC	Adj. R²	p	DF	SP (± SE)	SL Minor (± SE)	SL Major (± SE)	ANOVA between models
A	Pooled	Segmented	-680.01	62.09	0.71	<0.01	759	3.10 (± 0.01)	1.78 (± 0.11)	3.29 (± 0.15)	DF = 1, F = 34.34, P <0.01
		Linear	-617.92		0.69	<0.01	761				
B	MON	Segmented	-136.94	16.73	0.86	<0.01	105	3.13 (± 0.02)	1.92 (± 0.21)	3.64 (± 0.31)	DF = 1, F = 10.99, P <0.01
		Linear	-120.21		0.83	<0.01	107				
	BOF	Linear	-38.20	1.31	0.68	<0.01	80				DF = 1, F = 1.30, P = 0.28
		Segmented	-36.88		0.68	<0.01	78	3.04 (± 0.07)	1.77 (± 0.54)	2.74(± 0.31)	
	CRO	Segmented	-212.50	7.47	0.75		136	2.99 (± 0.03)	0.66 (± 0.69)	2.48 (± 0.15)	DF = 1, F = 5.80, P <0.01
		Linear	-205.04		0.73		138				
	TOC	Segmented	-107.99	1.07	0.70	<0.01	71	3.02 (± 0.05)	1.23 (± 0.37)	2.26 (± 0.30)	DF = 1, F = 2.48, P = 0.09
		Linear	-106.92		0.69	<0.01	73				
	FEL	Segmented	-455.77	35.36	0.77	<0.01	353	3.10 (± 0.01)	1.80 (± 0.14)	3.28 (± 0.19)	DF = 1, F = 20.57, P <0.01
		Linear	-420.41		0.74	<0.01	355				

AIC = Akaike Information Criterion score

ΔAIC = difference in AIC value relative to the top model

Adj. r^2 = adjusted r^2 value

DF = degree of freedom

SP (± SE) = estimated switch point value (± standard error)

SL = slope value, i.e., allometric coefficient

See FIGURE 1 for explanation of sites abbreviations.
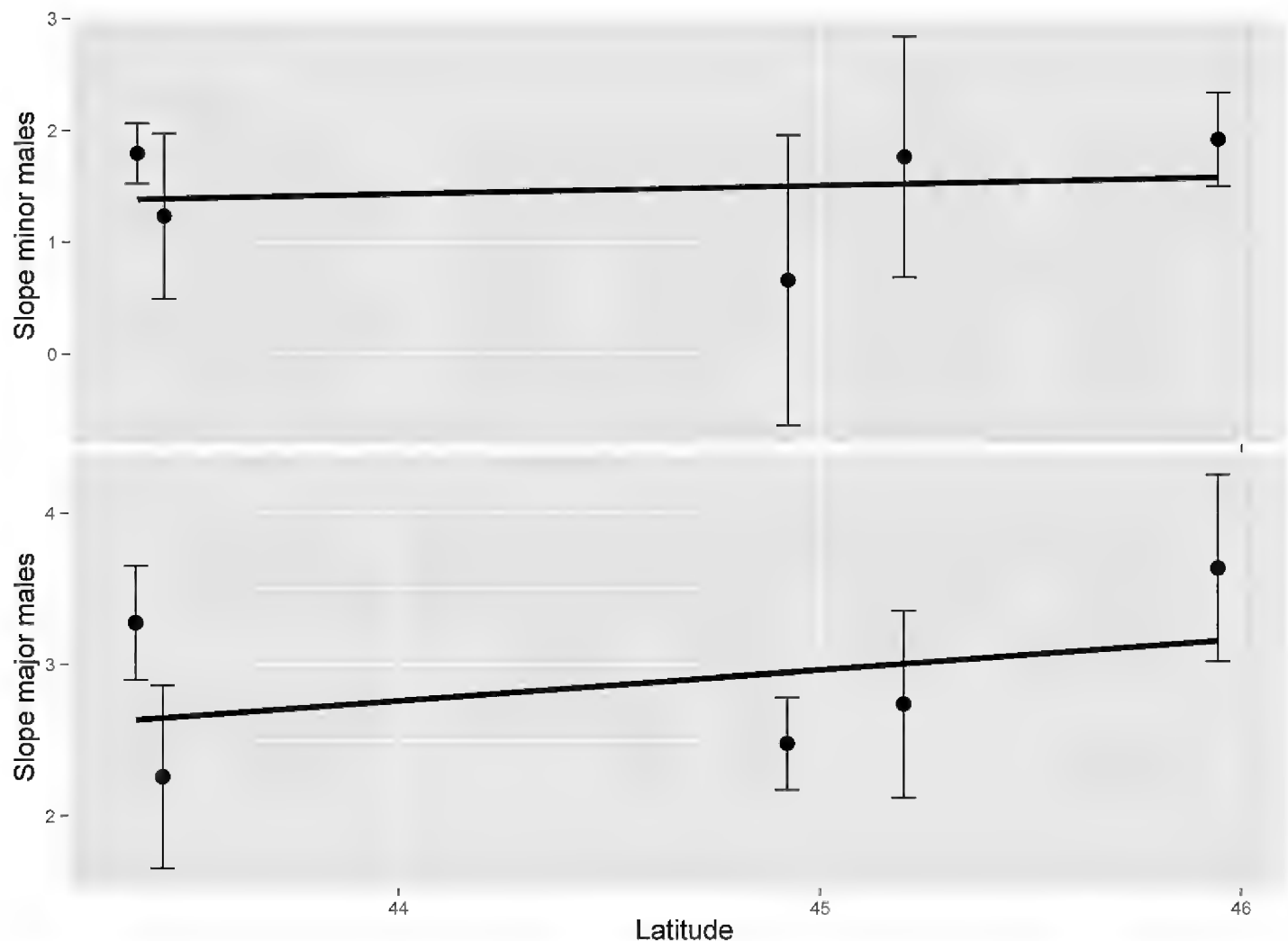
Figure 4. Allometric coefficient (± 95% confidence intervals) between weapon (LnML) and body size (LnEL) in relation to latitude for minor males and major males of *Lucanus cervus*.

The overall latitudinal cline for the allometric coefficients for both male morphs is illustrated in the scatterplots with superimposed trend lines (Figure 4). All the biometric variables exhibited a latitudinal variation, according to the result of the Kruskal-Wallis H test (BM: hc = 49.89, p< 0.01; ML: hc = 117.83, p< 0.01; EL: hc = 25.28, p< 0.01). The Dunn's test for multiple comparison showed that the less variable trait was EL, with a significant variation (p< 0.01) only between the population of TOC and the northernmost location (MON). Both BM and ML exhibited a significant variation amongst sites at the extremes of the examined latitudinal range (Bonferroni corrected p values: $BM_{MONvsFEL}$< 0.01; $BM_{MONvsTOC}$< 0.01; $BM_{BOFvsFEL}$< 0.01; $BM_{BOFvsTOC}$< 0.01; $ML_{MONvsFEL}$< 0.01; $ML_{MONvsTOC}$< 0.01; $ML_{BOFvsFEL}$< 0.01; $ML_{BOFvsTOC}$< 0.01; $ML_{CROvsTOC}$< 0.01; $ML_{CROvsFEL}$< 0.01). The PERMANOVA results indicated a strong dependence of the biometric variables on latitude (BM: f = 39.38, r^2 = 0.06, p< 0.01; EL: f = 14.45, r^2 = 0.02, p< 0.01; ML: f = 106.35, r^2 = 0.11, p< 0.01). The box-plots in Figure 5 A-C illustrated the variation in biometric rough variables amongst populations. The median value of BM varied from around 1.8g (TOC: 1.7g; FEL: 1.9g) of the southernmost populations to 2.3g (BOF: 2.4g; MON: 2.2g); medians of ML and EL ranged, respectively, from 9mm (FEL) to 11mm (MON) and from 21mm (TOC) to 23mm (MON).
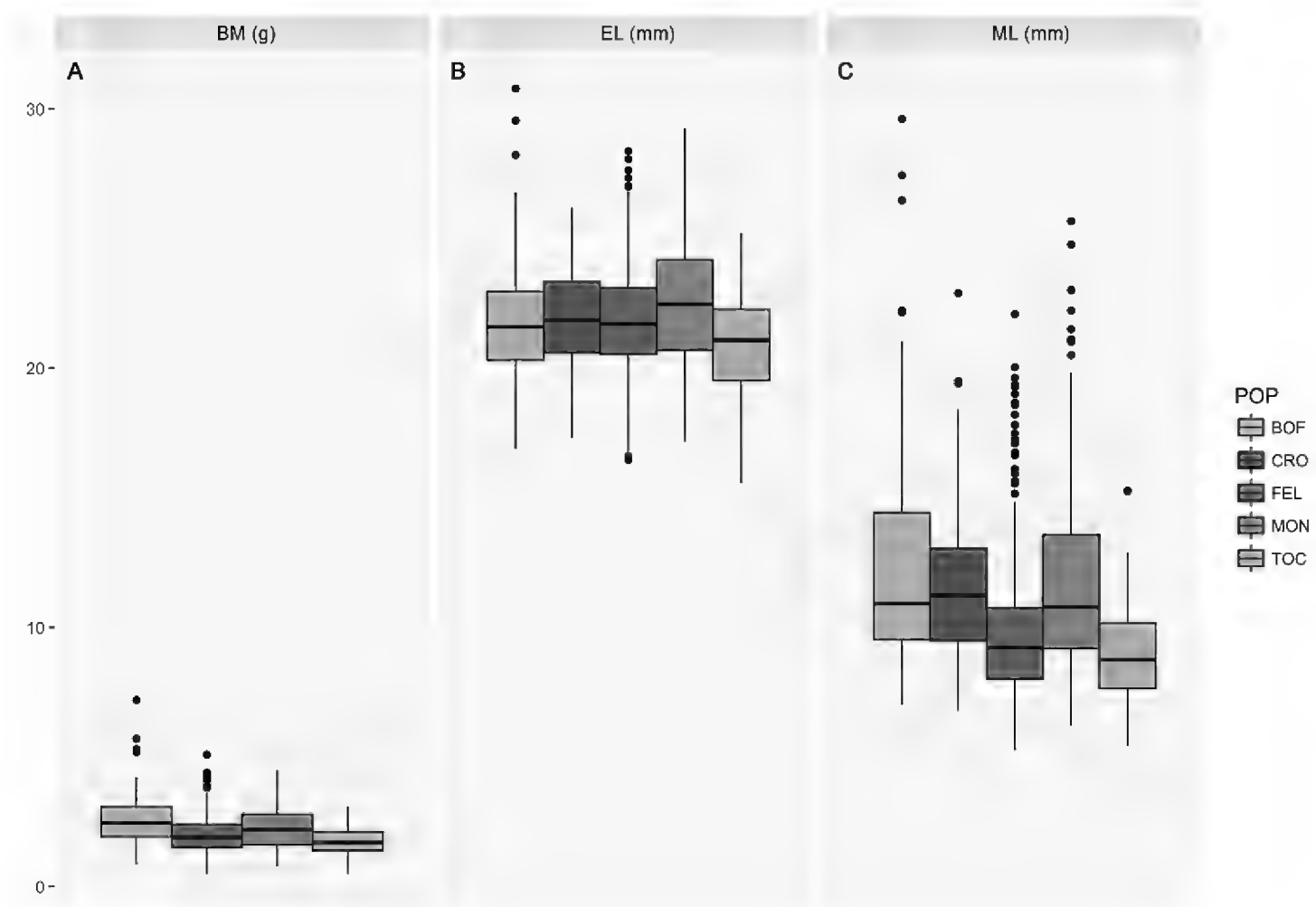
Figure 5. Box-plots for the variation in biometric rough variables, between populations: body mass (BM), elytron length (EL) and mandible length (ML). See Figure 1 for the acronyms of the study sites.

Phenology

The populations exhibited a significant variation in adult activity period (Kruskal-Wallis: $hc = 114.3$, $p < 0.01$). In particular, the comparison amongst sites highlighted a significant shift in the phenology between the sites close to the Alps (MON and BOF) and those close to the Apennines (CRO, TOC and FEL) (Mann-Whitney: $p_{MONvs-FEL} < 0.01$; $p_{MONvsTOC} < 0.01$; $p_{MONvsCRO} < 0.01$; $p_{BOFvsFEL} < 0.01$; $p_{BOFvsTOC} < 0.01$; $p_{BOFvsCRO} < 0.01$) and an insignificant difference for the other comparisons (Mann-Whitney: $p_{MONvsBOF} = 0.96$; $p_{TOCvsFEL} = 0.91$; $p_{TOCvsCRO} = 0.06$; $p_{FELvsCRO} = 0.36$).

Discussion

The results revealed a clear distinction between the studied sites, according to the combination of their climate variables. The northernmost site (MON) was shown to be the rainiest locality, with the lowest monthly temperatures (MIN, MEA and MAX). This is in accordance with the updated climate classification map of Kottek et al. (2006) which reports the Piedmont Prealps straddling two climate categories: snow climate, fully humid with cool summer (Dfc) and warm temperate climate, fully humid (Cf). The remaining sites (BOF, CRO, TOC and FEL), although varying in terms of aver-

age temperatures, did not differ significantly and were characterised by a dry summer period. Although BOF exhibited the highest summer temperatures and lowest winter temperatures, both northernmost sites show minimum temperatures below zero during winter. Although the humidity and the average monthly hours of sunshine could play an important role in characterising the study sites, the authors were unable to gather data for these variables. The overall trend in total precipitation indicated a correlation with latitude and, consequently, the dry summer period was more pronounced in the Po plain and along the Tiber valley compared to that recorded in MON.

The analysis of mandible allometry on the pooled dataset strengthens the results obtained by Romiti et al. (2015) clearly identifying two morphological classes in *L. cervus* males: minor and major. Minor males, although characterised by a positive allometry, presented a shallower allometric coefficient line (i.e., low slope value) compared to major males. Thus, at species level, the allometric trajectory undergoes an increase in slope with increase in body size. It is therefore concluded that *L. cervus* belongs to the species of the genus *Lucanus*, identified by Knell et al. (2004), which invest relatively more in weaponry compared to other *Lucanus* spp., characterised by relatively long mandibles, where the mandible length is clearly greater than half of the body length, for example, *L. laminifer* (Waterhouse, 1890) and *L. planeti* (Planet, 1899). This suggests that, in *L. cervus*, weapon lengthening is not affected by any depletion of resources by other body parts. The differential development of weaponry between minor and major males could be explained as a balance between the benefit derived from a given amount of investment and its cost (Gadgil 1972). The advantages of bearing enlarged weapons becomes particularly evident during stag beetles male-male interaction, giving an enhanced capacity for dislodging the opponent, both in interspecific and intraspecific contests (Hongo and Okamoto 2013, Lagarde et al. 2005), thus shortening the battle duration (Goyens et al. 2015a) and finally resulting in increased mating opportunities. However, exaggerated mandibles can be costly in terms of impairing locomotion and stability (Goyens et al. 2015b) and constraining flight ability (Goyens et al. 2015c). Despite the great investment in SSC by major males, Romiti et al. (2016) suggested that functional constraints, (for example, enhanced mechanical stress and reduced flight performance), may have shaped the mandibles of larger *L. cervus* males. Therefore, while some constraints maintain the mandible shape of major males, their lengthening is driven by increase in body size. The segmented regression is the first ranked model in four of the five analysed populations, probably indicating the existence of a genetically determined threshold size at which individuals switch from one morph to another. As with Painting et al. (2014), a latitudinal increase in overall body size of male stag beetles was found. This cline could be positively selected to reduce cooling rate (i.e., to increase the thermal inertia) at high latitudes, consistent with Bergmann's Rule. Despite investigating a narrow latitudinal range, the results obtained indicate a latitudinal cline in weapon allometry and phenology. Indeed, in studies over larger geographical ranges, latitude/altitude-independent confounding factors grow too numerous to reflect a true clinal trend (Shelomi 2012). Nevertheless, it would be interesting to investigate this phenomenon at European level to validate or refute the trend high-

lighted in the present study (cf. Box 1). In accordance with other studies on stag beetles (Rowland and Emlen 2009, Iguchi 2013, Romiti et al. 2016), the most variable trait is mandible length, both within and amongst populations. It is indeed well known that SSC express a huge phenotypic plasticity in both size and shape (Andersson 1982, Fitzpatrick 1997, Cuervo and Møller 2001). In contrast, elytron length, which could be interpreted as a measure of the post-prothoracic segments (mesothorax, metathorax and abdomen), did not show a great latitudinal variation, as was expected for a non-SSC. The mandible length exhibited the same latitudinal trend as body mass, increasing with latitude. Moreover, contrary to what has been demonstrated by Painting et al. (2014) for *L. barbicornis*, at high latitudes, the allometric slope line for major males increases. This variation in the scaling relationship (LnML vs LnEL) derives from a greater investment in weapon size at high latitude coupled with an equal investment in elytron length across the latitudinal range. These results further confirm that males of the European stag beetle are in an arms race, thus the more resources they gather during larval stage, the more they invest in weaponry. In other words, major males, who have grown larger in terms of body mass, have reduced resources for production and maintenance of SSC compared to minor males which did not attain the minimum body size to develop exaggerated weapons. In accordance with this hypothesis, the elevations analysis indicates that individuals of the same size, when belonging to latitudinally distant populations (i.e., northern vs southern), are equipped with more elongated mandibles as latitude (hence body mass) increases. It is worth noting that both larval growth conditions and feeding resources (Moczek 2002) have an effect on the developmental mechanisms that regulate the elaboration of exaggerated structures in holometabolous insects (Shingleton et al. 2008). Indeed, it has been demonstrated that in stag beetles, the development of SSC is closely associated with larval feeding conditions, the mediatory function of the juvenile hormone (Gotoh et al. 2011) and the expression of transcription factors encoded by double-sex genes (Gotoh et al. 2014). Larvae of the studied populations, described above, have grown in different woodland types (e.g., oak grove, chestnut) and have experienced different nutritional conditions. These differences, together with larval microhabitat characteristics, could be responsible for the variation in allometric slope and elevation which are highlighted amongst these populations. In fact, differences in slope and elevation for the scaling relationship between mandibles and body size were also recorded between sites which appeared to be comparable at a macroclimate scale (BOF, FEL and TOC). This led to the conclusion that variables such as deadwood type, decay trajectory (influenced by the way in which the tree died (Parks 1999)) and soil humidity could play an important role in the resulting allometric trajectories. However, it was not possible to separate the role of genetic variation from the local environmental features which influence larval development, SSC plasticity and physiological constraints. In-depth breeding experiments are required, conducted under different microclimate conditions using individuals from the same clutch and these clutches have to be representative of different latitudes. In fact, it has been demonstrated that different populations respond differently to climate changes (Pelini et al. 2012): they can be adapted to historically different conditions (Gilman et al. 2006,

Pelini et al. 2009, Angert et al. 2011) and have different abilities to cope with local environmental changes (Magnani 2009). However, as *L. cervus* has a long larval stage (3-6 years) which can vary significantly across its distribution (Harvey et al. 2011), this makes these breeding experiments difficult. A latitudinal shift in adult phenology of stag beetles has been identified, indicating an earlier mating season at high latitude. Saproxylic beetles, whose larvae live in and feed on the decaying wood (for example, dead roots, fallen branches and stumps of both living and dead trees), like *L. cervus*, could be sensitive during the larval and pupal stage to the temperature cycle (thermoperiod) experienced in their microhabitat. The thermoperiod is also likely to depend on microclimate characteristics such as humidity of the woodland and consequently dead wood, as well as ground temperature, all of which are influenced by canopy density and monthly hours of sunshine. In addition, the thermoperiod will be influenced by macroclimate variables which exhibit a latitudinal gradient. Latitudinal variation could contribute to life-stages shift amongst latitudes, thus directly influencing the larval development and/or setting constraints on the availability of food resources. As many aspects of ectotherm physiology are temperature dependent (Huey 1982), the variation in adult activity is likely correlated with behavioural thermoregulation. Male stag beetles actively search for mates while flying at dusk during the mating season (Campanaro et al. 2011, Chiari et al. 2014). Flying activity is closely correlated to ambient thermal conditions (Adams and Heath 1964, Heinrich 2013). It is suggested that the latitudinal shift in activity period could be positively selected to enhance the dispersal ability of males, making their thermoregulation more effective. Individuals at higher latitudes could emerge earlier to make the most of a short, cool and rainy summer period (MON) or, alternatively, to avoid a very hot summer (BOF) (climate category of the Po plain according to Kottek et al. (2006): Cfa, warm temperate, fully humid with hot summer).

Conclusions

The latitudinal cline in body size has been one of the most widely observed patterns in nature and has interested biologists for over 150 years. Animal body size, according to Bergmann's Rule, increases with latitude. By studying five populations which span the Italian distribution of the species, a geographical variation in mandible and body size in the stag beetle *L. cervus* was identified. These results indicate that males of this species invest relatively more in weaponry size at high latitudes, leading to a further exaggeration of this SSC. On the contrary, the size of the post-prothoracic segments did not exhibit a latitudinal cline. As well as defining the allometric coefficient slope for male weaponry, it was shown that the breeding season varies with latitude, beginning later on (late June) at lower latitudes. Characterisation and comparison of adult phenologies, besides being crucial for modelling the insects' response to climate change, provided valuable data for the conservation and monitoring of a threatened species and are also used as flagship and umbrella species for the conservation of saproxylic fauna in Europe.

Acknowledgements

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Appendix

Box 1. Stag beetle researchers are invited to send us morphometric data on *L. cervus*, including at least: mandible length, elytron length and body mass, measured as in Romiti et al. (2015). We will analyse and compile the results with the intention of submitting an article in January 2018, with all the contributors who submit more than 30 records accredited as co-authors.

Supplementary material I

Shapiro-Wilk normality test on biometric, phenological and climatic variable
Authors: Federico Romiti, Lara Redolfi De Zan, Sarah Rossi de Gasperis, Massimiliano Tini, Davide Scaccini, Matteo Anaclerio, Giuseppe Maria Carpaneto
Data type: statistical data
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/natureconservation.19.12681.suppl1

Supplementary material 2

Monthly total precipitations and temperatures

Authors: Federico Romiti, Lara Redolfi De Zan, Sarah Rossi de Gasperis, Massimiliano Tini, Davide Scaccini, Matteo Anaclerio, Giuseppe Maria Carpaneto

Data type: environment data

Link: https://doi.org/10.3897/natureconservation.19.12681.suppl2

Supplementary material 3

Allometric relationship between mandible (LnML) and elytron (LnEL) length for minor and major morph

Authors: Federico Romiti, Lara Redolfi De Zan, Sarah Rossi de Gasperis, Massimiliano Tini, Davide Scaccini, Matteo Anaclerio, Giuseppe Maria Carpaneto

Data type: statistical data

Link: https://doi.org/10.3897/natureconservation.19.12681.suppl3

Supplementary material 4

Allometric relationship between mandible (LnML) and elytron (LnEL) length of each population

Authors: Federico Romiti, Lara Redolfi De Zan, Sarah Rossi de Gasperis, Massimiliano Tini, Davide Scaccini, Matteo Anaclerio, Giuseppe Maria Carpaneto

Data type: statistical data

Link: https://doi.org/10.3897/natureconservation.19.12681.suppl4